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Development of Improved Defined Media for *Clostridium botulinum* Serotypes A, B, and E

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The minimal nutritional growth requirements were determined for strains Okra B and Iwanai E, which are representatives of groups I and II, respectively, of *Clostridium botulinum*. These type B and E strains differed considerably in their nutrient requirements. The organic growth factors required in high concentrations by the Okra B strain (group I) were arginine and phenylalanine. Low concentrations (≤0.1 g/liter) of eight amino acids (methionine, leucine, valine, isoleucine, glycine, histidine, tryptophan, and tyrosine) and of five vitamins (pyridoxamine, *p*-aminobenzoic acid, biotin, nicotinic acid, and thiamine) were also essential for biosynthesis. The 10 required amino acids could be replaced by intact protein of known composition by virtue of the bacterium's ability to synthesize proteases. Glucose or other carbohydrates were not essential for Okra B, although they did stimulate growth. Quantitatively, the most essential nutrients for Okra B were arginine and phenylalanine. In contrast, the nonproteolytic strain, Iwanai E (group II), did not require either arginine or phenylalanine. It required glucose or another carbohydrate energy source for growth and did not utilize arginine or intact protein as a substitute source of energy. Iwanai E utilized ammonia as a nitrogen source, although growth was stimulated significantly by organic nitrogenous nutrients, especially glutamate and asparagine. Iwanai E also required biosynthesis levels of seven amino acids (histidine, isoleucine, leucine, tryptophan, tyrosine, valine, and serine), adenine, and six vitamins (biotin, thiamine, pyridoxamine, folic acid, choline, and nicotinamide). Calcium pantothenate also stimulated growth. On the basis of the nutritional requirements, chemically defined minimal media have been constructed for *C. botulinum* serotypes A, B, E, and F (proteolytic). These media have been very useful in our laboratory for studying the physiology of growth and toxin production by *C. botulinum*.

The anaerobic sporeforming bacterium *Clostridium botulinum* is widely dispersed in soil and water and produces the most lethal protein neurotoxin known (ca. 10^{-8} mg is lethal for a mouse, and it is estimated that ingestion of 0.2 to 2 μg may kill a human) (24). The species *C. botulinum* is actually a collection of clostridial strains which have in common the synthesis of botulinal neurotoxin but which differ widely in many other phenotypic properties and in nucleic acid relatedness (2, 11, 25). Eight neurotoxin serotypes (A, B, C_1, C_2, D, E, F, and G) are recognized (26). *C. botulinum* is further separated into four physiological groups (I to IV) principally on the basis of its proteolytic capabilities (25). Group I strains are strongly proteolytic and readily digest casein or meat proteins, whereas group II strains are nonproteolytic or weakly proteolytic. These two groups also differ in several other properties, including heat resistance of spores, relative toxigenicity, maximum growth temperature, and tolerance to salt and low pH (15, 25). Groups I and II, comprising toxin types A, B, E, and F, are responsible for nearly all confirmed cases of human botulism (25, 26). With few exceptions (7, 18), group I strains have been implicated in every incident of infant botulism.

The *C. botulinum* strains responsible for human botulism (serotypes A, B, E, and the rare F) and the related organism *Clostridium sporogenes* are known to have complex nutrient requirements. They require at least eight amino acids and several vitamins (5, 6, 8–10, 12, 14, 17, 27). Despite numerous studies on *C. botulinum* which demonstrated the necessity for several growth factors, the individual nutritional requirements have not been quantitated in relation to cell growth. Minimal media are not presently used for studying this pathogen. The use of complex media, however, often imposes difficulties in the interpretation of physiological studies, such as in the pathways of the catabolism of individual amino acids (1). Moreover, the use of complex media precludes certain genetic manipulations, including the isolation of auxotrophs and some other mutants. In this study, we have identified the requirements for individual nutrients and have quantitated the levels needed for good growth of representative organisms in *C. botulinum* groups I and II. Identification and quantitation of the nutrient requirements have enabled us to construct minimal media for serotypes A, B, and E. Because we have found these media to be very useful in studying neurotoxin regulation and other aspects of *C. botulinum*, the experiments leading to their development and their final compositions are included here to assist other laboratories in the study of *C. botulinum*. Furthermore, the distinctive differences that we have found in the minimal nutrient requirements of groups I and II support the hypothesis that these two groups are phenotypically distantly related except for the common property of neurotoxin synthesis.

MATERIALS AND METHODS

Materials. Hungate tubes (16 by 125 mm) and butyl rubber stoppers for anaerobic culture were obtained from Bellco Glass, Inc., Vineland, N.J. An anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) was inflated with a prepared gas mixture (80% N_2, 10% CO_2, 10% H_2). Chemical reagents used were commercial products of the highest grade available. All amino acids (L isomers) and vitamins were from Sigma Chemical Co., St. Louis, Mo. A complete

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vitamin mix, minimal essential medium, consisting of the following components (in milligrams per liter): NaCl, 8,500; calcium pantothenate, 100; choline chloride, 100; folic acid, 100; *i*-inositol, 200; nicotinamide, 100; pyridoxal hydrochloride, 100; riboflavin, 10; and thiamine hydrochloride, 100, was obtained from GIBCO Laboratories, Grand Island, N.Y. It was supplemented with vitamin B_{12} (0.2 mg/liter) and *p*-aminobenzoic acid (0.4 mg/liter) and diluted 100-fold upon addition to the medium.

Bacterial strains. The *C. botulinum* (types A to G, 34 strains) and *C. sporogenes* (11 strains) strains used in this study are listed in Table 1. The Okra B proteolytic strain is a stock culture from H. Sugiyama's laboratory (4, 20). It was used primarily for the study of group I nutrient requirements, but other type A and B strains were also tested in conjunction, including Hall A (ATCC 3502) and 213B. Three type E strains (Alaska, Minnesota, and Iwanai [27]) were used for the determination of group II nutrient requirements. The strains were periodically plated in an anaerobic glove box onto TPGY agar (composition [in grams per liter]: Trypticase peptone [BBL Microbiology Systems, Cockeysville, Md.], 50; Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 5; yeast extract, 20; glucose, 4) containing 0.1% cysteine hydrochloride as a reducing agent. The morphology of streaked cultures was examined, and individual colonies were picked, grown for five days in TPGY medium, and tested for neurotoxin formation by injecting culture fluid intraperitoneally into mice and noting the time to death (13, 23). In this toxin assay, the time to death of mice injected intraperitoneally with culture fluid correlates logarithmically with the dose required to kill 50% of a population of mice assayed by the quantal method. Two mice were used for each determination. Mice used as controls were protected immunologically with type-specific antisera. Purified cultures of strains and their spores were stored at −20°C in TPGY medium or at −80°C in 50% glycerol.

Preparation of media and cultivation of bacteria. Cultures of *C. botulinum* strains were removed from the freezer (−80°C) and inoculated (1%, vol/vol) into Todd-Hewitt broth or TPGY medium. These cultures were used as inoculum for the minimal media as described in Results and were subcultured (1%, vol/vol) into synthetic media.

The chemically defined media for group I or group II strains (MI and MII, respectively) (see Table 4) were prepared by combining the following groups of nutrients: (i) a 10-fold-concentrated solution of A salts {$NaH_2PO_4 \cdot H_2O$, K_2HPO_4, and K_2SO_4 [or $(NH_4)_2SO_4$; see Results]}, (ii) a 10-fold-concentrated amino acid solution (for MI: arginine, phenylalanine, methionine, tryptophan, valine, glycine, histidine, isoleucine, leucine, and tyrosine; for MII: histidine, isoleucine, leucine, tryptophan, valine, serine, and tyrosine), and (iii) resazurin and 770 ml of distilled H_2O. After the nutrients were mixed, and just before they were dispensed into tubes, $NaHCO_3$ and cysteine hydrochloride were added. The tubes were capped after they were sparged with N_2, and they were then autoclaved for 15 min at 121°C. A 100-fold-concentrated trace B salt solution ($FeSO_4 \cdot 7H_2O$, ZnCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $MnCl_2$) was autoclaved separately, combined with a filter-sterilized 100-fold-concentrated vitamin solution (for MI: *p*-aminobenzoic acid, pyridoxamine, biotin, nicotinic acid, and thiamine; for MII: biotin, thiamine, pyridoxamine, folic acid [dissolved in 0.01 N NaOH], choline, and nicotinamide), and finally combined with an autoclaved 100-fold-concentrated carbon source solution; after it was autoclaved, this mixture was added to the tubes by use of a syringe inserted through the butyl rubber septa. The concentrations of the nutrients in MI and MII are listed in Table 4.

TABLE 1. Bacterial strains used in this study

Strain	Group	Toxin serotype	Source[a]
C. botulinum	I	A	
Hall A			H. Sugiyama
Hall A			ATCC
62A			H. Sugiyama
A109-1			H. Sugiyama
A109-2			H. Sugiyama
A112			H. Sugiyama
A73			H. Sugiyama
25763			ATCC
Okra B	I	B	B. R. DasGupta
Okra B			H. Sugiyama
13983B			H. Sugiyama
169B			H. Sugiyama
113B			H. Sugiyama
213B			H. Sugiyama
Beans B			H. Sugiyama
17844			ATCC
Lamanna B			H. Sugiyama
17B	II	B	ATCC
17B			H. Sugiyama
2129B			H. Sugiyama
2048	III	C	H. Sugiyama
468			J. Price
2054	III	D	H. Sugiyama
Alaska E	II	E	H. Sugiyama
Minnesota E			H. Sugiyama
Iwanai E			H. Sugiyama
Beluga E			D. Gombas
Whitefish E			D. Gombas
Langeland F	I	F	H. Sugiyama
VPI 4257 F			H. Sugiyama
83F	II	F	H. Sugiyama
33263	IV	G	ATCC
2738			H. Sugiyama
C. sporogenes			
59123			H. Sugiyama
1026			H. Sugiyama
4472			H. Sugiyama
4479			H. Sugiyama
1106-A			H. Sugiyama
1106-B			H. Sugiyama
PA3679			J. Hanlin
3584			ATCC
19404			ATCC
4439			H. Sugiyama
4472			H. Sugiyama

[a] ATCC, American Type Culture Collection.

Cells were grown at 35°C (group I) or 30°C (group II) in anaerobic Hungate tubes with a N_2 atmosphere. Growth was measured by monitoring the optical density at 660 nm (OD_{660}) in a Bausch & Lomb Spectronic 20 spectrophotometer; 1 mg (dry weight) of cells per ml corresponds to ca. 1.08 optical density units. Inocula for sequential transfers consisted of 1% (vol/vol) exponentially growing cells.

Purification of casein. Casein (Sigma) was freed of contaminating substances by the purification protocol of Rosenberg et al. (22).

RESULTS

Amino acid and vitamin requirements for growth. *C. botulinum* proteolytic type B (Okra B; group I) and three strains of nonproteolytic type E (Alaska E, Minnesota, and Iwanai; group II) were used to initially determine the nutritional requirements for growth. Since all three type E strains were found to have nearly identical requirements, the growth responses of strain Iwanai are described mainly in Results. After the initial determination, several other group I and II strains were tested for their ability to grow in the respective media (see below).

C. botulinum Okra B adapted readily to a synthetic medium containing a multivitamin mix (minimal essential medium; see Materials and Methods), a 20-amino-acid mixture prepared in proportion to imitate casein, and glucose (1%) as its principal organic constituents. The cells reached an OD_{660} of ca. 1, demonstrating that the medium satisfied the nutritional needs of Okra B. The type E strains did not grow in the same synthetic medium, although they did grow to an OD_{660} of 0.6 to 0.8 in the synthetic 20-amino-acid–8-vitamin D-Y medium of Gullmar and Molin (5, 6). Therefore, D-Y medium was used for strain Iwanai E as a complete basal medium, and individual nutrients were tested to determine whether they were essential.

To determine amino acid requirements, cells were transferred to a medium deficient in 1 of the 20 amino acids. The Okra B culture inoculated into arginine-deficient medium failed to grow on this first transfer. Second and third transfers (1% [vol/vol] inocula) were carried out with the other amino acid-deficient media. After two transfers, we observed that eight amino acids were essential for growth:

TABLE 2. Growth of *C. botulinum* Okra B and Iwanai E in synthetic media deficient in a specific amino acid[a]

Amino acid omitted	Maximum growth[b] (A_{660}) of:	
	Okra B	Iwanai E
L-Arginine[c]	0.03	0.65
L-Asparagine	0.96	0.68
L-Glutamic acid	1.0	0.84
Glycine	0.87	0.72
L-Histidine	0.66	0.21 (0.20)[d]
L-Isoleucine	0.08	0.01
L-Leucine	0.10	0.02
L-Methionine	0.08	0.89
L-Phenylalanine	0.01	0.81
L-Serine	1.6	0.20 (0.005)[d]
L-Tryptophan	0.04	0.02
L-Tyrosine	0.08	0.035
L-Valine	0.04	0.02
Complete medium	1.0	0.90

[a] Neither Okra B nor Iwanai E showed requirements for L-alanine, L-aspartic acid, L-cysteine, L-glutamine, L-lysine, L-proline, or L-threonine. Growth (A_{660}) when these amino acids were omitted was ≥1.0 for Okra B and ≥0.45 for Iwanai E.

[b] Optical density was recorded during two transfers in the synthetic medium deficient in a specific amino acid. The values given correspond to 24 and 88 h of growth for Okra B and Iwanai E, respectively. The inoculum was 1% (vol/vol) in late log phase.

[c] Recorded after the first transfer.

[d] Recorded after the third transfer (95 h of growth).

TABLE 3. Vitamin requirements of *C. botulinum* Okra B and Iwanai E

Vitamin omitted	Maximum growth (OD_{660}) after two (Okra B) or three (Iwanai E) transfers	
	Okra B	Iwanai E
Pantothenic acid (Ca salt)	1.0	0.65
Choline chloride	1.1	0.14
Nicotinic acid	0.83	0.80
Nicotinamide	ND[a]	0.01
Pyridoxamine	0.05	0.70
Pyridoxine	ND[a]	0.90
p-Aminobenzoic acid	0.81	0.80
Biotin	0.14	0.24
Thiamine	0.16	0.02
Folic acid	ND[a]	0.0
Complete medium	1.0	0.75

[a] ND, Not done.

arginine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine (Table 2). However, a synthetic medium prepared with these eight amino acids failed to sustain growth on consecutive transfers. The addition of histidine and glycine, which are required in only small amounts (Table 2), provided for good growth through five consecutive transfers. Others investigators (17) have also recognized requirements for 10 amino acids in the group I *C. botulinum*; therefore, this finding was not pursued further with other strains.

Similarly, the amino acid requirements for the type E strains were determined by elimination of individual amino acids for the D-Y medium. After the first transfer (72 h of growth), all three strains grew to an OD_{660} of ~0.60 when any one amino acid was omitted, except for tryptophan—when it was deleted, the final optical density was <0.2. After second and third transfers, fairly high levels of isoleucine, leucine, tryptophan, tyrosine, and valine were needed. Lesser amounts of histidine and serine were required. In contrast to the other type E strains, the Alaska type E strain did not require tyrosine for growth. In summary, the proteolytic strain Okra B required 10 amino acids and the type E strains required 7.

Next, we defined the vitamin requirements of Okra B by single-vitamin-elimination experiments in a salts medium supplemented with the 10 required amino acids and glucose as an energy source. After two transfers, requirements for pyridoxamine, thiamine, and biotin were apparent (Table 3). For maximum cell densities on successive subcultures, it was necessary to supplement the medium additionally with *p*-aminobenzoic acid and nicotinic acid. To further check the vitamin requirements of the group I *C. botulinum* (since these requirements are more variable than amino acid requirements), we compared the growth response of the Hall strain (serotype A) on elimination of individual vitamins from the mix derived for Okra B. After two successive transfers, requirements for *p*-aminobenzoic acid, biotin, and thiamine were apparent for strain Hall A. Hall A growth was slower in the absence of pyridoxamine and nicotinic acid, but the final optical density after 3 days (~1.0) was more than 90% that produced by growth in the complete five-vitamin mix required for Okra B. Thus, there was variation among the proteolytic organisms with regard to vitamins, but the combination of all five vitamins provided for growth of both A and B serotypes.

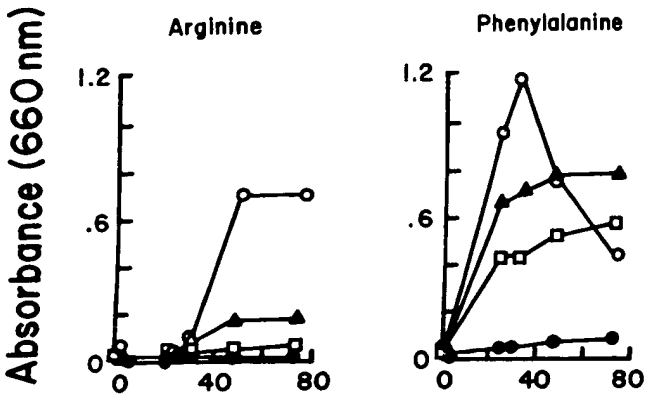

FIG. 1. Catabolic amino acid requirements of *C. botulinum* Okra B. The data are presented as growth (OD_{660}) on the ordinate as a function of time (in hours). The left and right panels show on the abscissa arginine and phenylalanine concentrations, respectively, at 0 ( ), 0.01 (□), 0.1 (▲), or 1 (○) g/liter.

To determine the vitamin requirements of the type E strains, we first examined whether the high concentration of sodium acetate (2.43 g/liter) in D-Y medium was essential for growth and whether it could be replaced with lipoic acid. The removal of acetate from the medium was also desirable because the final pH of D-Y medium was ca. 5.0; thus, cell density was probably limited by the acidic environment. We found that the high concentration of acetate was not necessary in the phosphate salt basal medium described in Results. After the elimination of acetate, the vitamin requirements of Iwanai E were determined by the elimination of individual vitamins from a complete mix. We found that Iwanai E required for growth choline, nicotinamide, biotin, thiamine, and folic acid. Nicotinic acid and *p*-aminobenzoic acid could not substitute for nicotinamide and folic acid, respectively. The elimination of both pyridoxine and pyridoxamine resulted in poor growth. The Alaska E strain had requirements identical to those of strain Iwanai E; however, the Minnesota strain required in addition calcium pantothenate, and the other strains were also stimulated by this growth factor. This extra requirement should be considered in encouraging the growth of certain nonproteolytic type E organisms. None of the strains appeared to require lipoic acid, an acetate replacement factor.

Biosynthetic and catabolic requirements for amino acids. The experiments described above show that *C. botulinum* Okra B and Iwanai E require for growth 10 and 7 amino acids, respectively. To distinguish biosynthetic requirements from requirements for amino acids needed in large quantities for catabolism (e.g., for generation of energy or nitrogen or to act as hydrogen acceptors), we determined the growth yields of cells in media containing various levels of the essential amino acids. Two patterns emerged for the Okra B strain. (i) Arginine and phenylalanine were required in concentrations much higher (≥1,000 mg/liter) than were needed for biosynthesis alone, while (ii) the remaining eight required amino acids were required only at low concentrations (≤100 mg/liter). The yield determinations for arginine and phenylalanine (Fig. 1) indicated that for maximum growth arginine is required at ≥3 g/liter and phenylalanine is required at ≥1 g/liter. In contrast, the three type E strains required each of their entire complement of amino acids at the relatively low level of ≤100 mg/liter. Therefore, the required amino acids for type E strains probably do not provide for cellular metabolism other than protein biosynthesis.

TABLE 4. Minimal medium compositions for *C. botulinum* groups I and II

Component	Concn (amt/liter) in: MI (for group I, proteolytic)	MII (for group II, nonproteolytic)
$NaH_2PO_4 \cdot H_2O$	4.7 g	4.7 g
K_2HPO_4	11.14 g	11.14 g
$(NH_4)_2SO_4$	NR[a] (NH_4^+)	2.64 g
K_2SO_4	3.48 g	
$FeSO_4 \cdot 7H_2O$	0.28 mg	0.28 mg
$ZnCl_2$	0.135 mg	0.135 mg
$CaCl_2 \cdot 2H_2O$	1.47 mg	1.47 mg
$MgSO_4 \cdot 7H_2O$	74.0 mg	74.0 mg
$MnCl_2$	0.2 mg	0.2 mg
Resazurin[b]	2.0 mg	2.0 mg
$NaHCO_3$[b]	1.0 g	1.0 g
Arginine	3.0 g	NR
Phenylalanine	1.0 g	NR
Methionine	0.1 g	0.1 g
Histidine	0.1 g	0.1 g
Isoleucine	0.1 g	0.1 g
Leucine	0.1 g	0.1 g
Glutamate	NR	0.5 g[c]
Glycine	0.1 g	0.1 g
Tryptophan	0.1 g	0.1 g
Tyrosine	0.05 g	0.05 g
Valine	0.1 g	0.1 g
Serine	NR	0.1 g
Cysteine hydrochloride[b]	1.0 g	1.0 g
p-Aminobenzoic acid	0.4 mg	NR
Biotin	0.2 mg	0.2 mg
Thiamine	0.4 mg	0.4 mg
Pyridoxamine	1.0 mg	1.0 mg
Nicotinic acid	1.0 mg	NR
Folic acid	NR	0.25 mg
Choline	NR	50 mg
Calcium pantothenate[c]	NR	50 mg[c]
Nicotinamide	NR	1.0 mg
Glucose	NR[c]	10 g
Adenine[c]	NR	0.01 g[c]
Sodium acetate[c]	NR	1.0 g[c]

[a] NR, Not required.
[b] Not required specifically; used as a reducing agent (cysteine hydrochloride), a source of CO_2 ($NaHCO_3$), and a redox indicator (resazurin).
[c] Stimulates growth but is not absolutely required.

Construction of defined minimal media. On the basis of the amino acid and vitamin requirements demonstrated for Okra B and Iwanai E, minimal synthetic media containing the minimum number of nutrients for growth were prepared by using inorganic salts (see Materials and Methods), amino acids, vitamins, and glucose at appropriate concentrations (Table 4). This medium supported excellent growth of Okra B and several other group I strains (see below); the OD_{660} consistently reached 1.0 to 1.3, and doubling times were ca. 2 to 3 h. However, the minimal medium designed for the type E strains reproducibly supported growth after 2 to 4 days to an OD_{660} of only 0.3 to 0.6. We felt that growth could be improved, and so we tested the following nutritive factors for their influence on the growth of the type E strains: nitrogen sources (asparagine, glutamine, glutamate, Casamino Acids, isoleucine, phenylalanine, and aspartic acid, each separately added at 0.5 g/liter), key metabolic intermediates (α-ketoglutarate, oxaloacetate, and citrate, each at 0.2 g/liter), nucleotide precursors (adenine, 0.01 g/liter, or a

TABLE 5. Carbon sources supporting growth of *C. botulinum* Okra B and Iwanai E in minimal media

Carbon source[a]	Maximum growth (OD_{660}) of:		Final pH[b]	
	Okra B	Iwanai E	Okra B	Iwanai E
None	0.38	0.01	7.18	6.96
D-Fructose	0.73	0.78	6.50	5.91
D-Glucose	0.90	0.87	6.47	5.72
Glycerol	0.59	0.07	6.92	6.90
Maltose	0.83	0.84	6.40	5.85
D-Mannose	0.29	0.90	7.14	5.86
Pyruvate	0.56	0.05	6.89	6.90
D-Ribose	0.31	0.84	7.03	5.88
Sorbitol	0.67	0.78	6.69	5.97
Sucrose	0.60	0.70	7.03	5.67
L-Arginine	ND[c]	0.01	ND	6.94
L-Ornithine[c]	0.18	ND	ND	ND
Starch	1.0	0.68	6.50	6.01
Casein	0.65	0.01	6.78	6.97

[a] Carbon sources were autoclaved separately and added at 10 g/liter. Glycerol and pyruvate (filter sterilized) were provided at 20 g/liter. The inoculum was developed in minimal medium with no sugar source. Growth was measured after a second transfer to medium containing the various carbon sources. Carbon sources giving insignificant growth for both strains included L-arabinose, inositol, lactose, mannitol, L-rhamnose, and D-xylose.
[b] Initial pH was 7.15 (Okra B) or 6.95 (Iwanai E).
[c] Substitution of 3 g of ornithine per liter for arginine and glucose.
[d] ND, Not done.

mixture of purines, 0.05 g/liter), and various vitamin-related factors (acetate, 1 g/liter; thioctic acid, 0.01 g/liter; and a 2× complete vitamin mix). Growth was approximately twofold stimulated by nitrogen-containing compounds, including asparagine, glutamate, glutamine, and Casamino Acids. These results suggested that the nonproteolytic strains were partially limited by the availability of nitrogen, which could not be sufficiently supplied in the form of ammonium ion. Calcium pantothenate, adenine, and either acetate or lipoic acid also stimulated growth 1.5- to 2-fold. We next performed an experiment in which we prepared a mixture of the stimulatory nutrients (asparagine, glutamine, glutamate, calcium pantothenate, adenine, acetate, and thioctic acid); individual nutrients were eliminated, and the growth response was measured. This experiment showed that L-glutamate, calcium pantothenate, adenine, and acetate were the most important supplementary nutrients. Asparagine also stimulated growth, but not as much as glutamate did. Although these five supplementary nutrients were not absolutely essential for the three type E strains tested, they stimulated growth significantly and are routinely added to the medium to promote good growth (Table 4). Growth in this supplemented medium consistently yielded optical densities of ca. 0.9 to 1.1 in 24 to 48 h, with doubling times of 2 to 4 h.

Influence of carbon and energy sources on growth. To evaluate the carbon and energy sources utilized by Okra B and Iwanai E in minimal media, we replaced glucose with various sugars, polysaccharides, and nitrogenous compounds. When glucose was omitted from MI, growth still occurred to a significant optical density of ca. 0.4 (Table 5). However, when arginine or phenylalanine was also omitted no growth occurred. These data suggest that type B *C. botulinum* can obtain energy and carbon from arginine and possibly from phenylalanine. However, even when phenylalanine was present, relatively little growth occurred when ornithine was substituted for glucose and arginine (Table 5). Therefore, arginine (and not phenylalanine) appears to be providing most of the energy for the cells. Other group I strains, including Hall A and 213B, showed the same response.

A number of carbohydrates and related compounds provided energy for Iwanai E and Okra B (Table 5) in minimal media. Both organisms grew well with fructose, glucose, maltose, sorbitol, sucrose, and starch. Okra B, but not Iwanai E, was stimulated by glycerol and pyruvate. In contrast, the type E cultures, but not Okra B, grew well on mannose and ribose. Although not tested with Okra B, the type E strains did not utilize cellobiose or galactose. The three type E strains were all tested for carbon source utilization, and each was uniform in its assimilation abilities. When the glucose concentration was tested from 0 to 50 g/liter, the optimum quantity was 10 g/liter. Casein or arginine provided carbon and energy for Okra B but not for the type E strains. Thus, the proteases secreted by Okra B allowed the digestion of purified casein and the acquisition of required amino acids. The type E cultures did not grow when arginine replaced glucose (Table 5). Growth in MI or MII did not appear to be limited by acidity since the final pH was ≥6.4 for Okra B and ≥5.7 for Iwanai E. The higher final pH values in the Okra B cultures may have been due in part to the release of ammonia from arginine.

Composition of the minimal medium and growth of various strains. The compositions of the chemically defined minimal media are listed in Table 4. The major organic nutrients required for the proteolytic Okra B strain in MI are arginine and phenylalanine, and growth is stimulated by glucose although a sugar is not essential to obtain significant growth. *C. botulinum* Okra B is fastidious and also requires for growth trace quantities of eight amino acids and five vitamins. The nonproteolytic type E strains require for growth a fermentable carbon source, ammonium ion, seven trace amino acids, and six vitamins. Growth of the type E strains is stimulated greatly by a supplementary source of organic nitrogen (glutamate or asparagine), adenine, calcium pantothenate, and acetate. Examination of the compositions of MI and MII media implies that growth of proteolytic Okra B is limited most by the availability of arginine and phenylalanine. In contrast, the type E strains are limited by the availability of a fermentable energy source and a source of organic nitrogen. Most of the nitrogen needed for growth of Okra B is supplied by arginine since excellent growth is obtained in MI when phenylpyruvate is substituted for phenylalanine (data not shown). Moreover, the growth rate of Okra B is not changed in MI when ammonium ion is provided in the medium (data not shown).

We tested the abilities of several group I and II *C. botulinum* strains to grow in the minimal media (Table 6). Numerous proteolytic group I strains of serotypes A, B, and F grew well in MI, and the five type E strains also grew well in MII. Unexpectedly, the nonproteolytic type B strains grew in MI but not in MII. These strains were also shown to require arginine for growth. Also, the nonproteolytic F strain tested grew poorly in MII and lysed very rapidly. Thus, the medium developed for type E appears to be specific for this serotype of *C. botulinum*.

Neurotoxin formation in minimal media. The formation of neurotoxin was determined for group I and II strains after growth for 5 days in the respective minimal media (Table 7). The titers ranged from 6×10^2 to 8×10^4 50% minimum lethal doses (mouse) per ml, depending on the strain tested. These titers are ca. 5 to 50 times less than those usually obtained in complex toxin production media (Table 7). Other

TABLE 6. Growth of various *C. botulinum* strains in minimal media

C. botulinum strain[a]	Group designation	Growth (OD_{660})[b] in:	
		MI	MII
Hall A (ATCC 3502)	I	0.96	0.01
62A	I	1.0	0.03
A109	I	1.1	0.01
C. sporogenes 59123	I	0.69	0.02
C. sporogenes 1026	I	0.93	0.03
C. sporogenes 4472	I	1.0	0.01
Okra B	I	0.96	0.02
169B	I	0.94	0.03
113B	I	0.92	0.01
17B (ATCC 25765)	II	0.95	0.02
2129B	II	0.82	0.03
Alaska E	II	0.03	1.05
Minnesota E	II	0.03	0.9
Iwanai E	II	0.01	0.85
Beluga E	II	0.01	0.98
Whitefish E	II	0.02	0.72
Langeland F	I	0.97	0.04
VPI 4257 F	I	1.0	0.02
83F	II	0.02	0.30

[a] Representative strains are presented in the table. We also tested for growth in MI of several other group I strains, all of which grew well: *C. sporogenes* (8 of 8 strains), *C. botulinum* type A (8 of 8), and type B proteolytic (9 of 9). Only the better-recognized strains are presented in the table.

[b] Growth is recorded for a second transfer in MI or MII.

TABLE 7. Toxin titers[a] of representative *C. botulinum* and *C. sporogenes* strains cultured in MI or MII minimal medium

Strain	Neurotoxin titer (MLD_{50}/ml)
C. sporogenes 1026	None
C. botulinum 169B	4.0×10^4
C. botulinum Okra B	8.0×10^4
C. botulinum Okra B (TPGY)[b]	5.0×10^5
C. botulinum 62A	1.0×10^3
C. botulinum type F, VPI 4257	4.0×10^3
C. botulinum Alaska E	8.0×10^4
C. botulinum Alaska E (TPGY)[b]	5.0×10^4
C. botulinum Iwanai E	3.0×10^2
C. botulinum Iwanai E (TPGY)[b]	8.0×10^3
C. botulinum Minnesota E	1.0×10^5
C. botulinum Minnesota E (TPGY)[b]	6.0×10^2

[a] Toxin titer (50% minimum lethal dose per ml [MLD_{50}/ml]) was determined by time to death of 18- to 20-g white mice after intraperitoneal injection (13, 24). See Materials and Methods for a description of the procedure.

[b] TPGY, Complex toxin production medium.

investigators have also observed low toxin titers in synthetic media (9, 12, 17).

DISCUSSION

The objective of the present study was to define and compare the minimal organic nutrient requirements for growth and toxigenesis by *C. botulinum* Okra B and Iwanai E and to develop defined minimal media that support good growth of *C. botulinum* groups I and II (25). The requirements for these two *C. botulinum* groups are strikingly different. For Okra B (group I), we demonstrated that several amino acids and vitamins are required in trace quantities, while only two organic nutrients, arginine and phenylalanine, are needed in high concentrations. Other investigators have recognized the need for arginine by *C. botulinum* group I (19, 21). Arginine is probably degraded by the arginine deiminase pathway to provide carbamoyl phosphate for energy production, ornithine for cell carbon, nitrogen, and polyamines, and carbamoyl phosphate for pyrimidine synthesis (21). Evidence obtained by Costilow and Cooper (3) suggests that arginine may be further catabolized partially to glutamate (1), a key nitrogen compound for biosynthesis (16). We have found that group I strains of *C. botulinum* contain unusually high levels of NAD^+-L-glutamate dehydrogenase (B. A. Hammer and E. A. Johnson, submitted for publication). This enzyme is absent or present at very low concentrations in group II strains. Therefore, arginine may be metabolized largely to glutamate and deaminated oxidatively to α-ketoglutarate, thus serving as an amino acceptor for transaminations in the proteolytic *C. botulinum*. The high level of phenylalanine required may serve as a hydrogen acceptor since we found that phenylpyruvate substitutes for it effectively and is reduced to phenylacetic acid (data not shown). Moreover, the conversion of arginine to glutamate may contribute to the well-known ability of group I strains to withstand increased osmotic stress, since glutamate and other related amino acids provide protection under increased osmolarity in certain bacteria. The liberation of ammonia from arginine could also maintain a suitable internal pH, which might help explain the ability of the proteolytic *C. botulinum* to withstand lower pH values than the nonproteolytic organisms withstand (15).

The requirements for arginine and phenylalanine suggest that *C. botulinum* Okra B has evolved mechanisms to effectively scavenge these two nutrients from its growth environment. We observed that *C. botulinum* can obtain arginine and phenylalanine from intact casein through protease activity. Protease synthesis and/or activity is lowered in minimal media containing high concentrations of arginine (S. Curtis and E. Johnson, manuscript in preparation). This finding illustrates the usefulness of minimal media in studying phenotypes of *C. botulinum*.

In contrast to Okra B and other group I strains tested, the nonproteolytic type E strains do not require arginine or phenylalanine. They do require a sugar or another carbohydrate to provide energy. The growth of the type E strains also seems to be limited by the availability of an organic source of nitrogen, such as glutamate. Unless a nitrogenous compound is provided, growth does not exceed an OD_{660} of 0.5. Therefore, the growth of certain nonproteolytic *C. botulinum* type E strains may be limited by the synthesis or availability in the environment of central nitrogenous metabolites. Iwanai E and two other type E strains also require seven amino acids, including several required by Okra B (histidine, isoleucine, leucine, tryptophan, tyrosine, and valine).

While examining various strains for growth in MI or MII, we observed unexpectedly that the two nonproteolytic type B strains examined grew in MI but not in MII (Table 6). This is surprising since the nonproteolytic type B strains are commonly classified in group II and have been shown to be saccharolytic (19) and to have ribosomal nucleic acid homology with other group II strains (11). However, we tested independent sources of strain 17B, including cultures from the Food Research Institute and an independent source obtained fresh from the American Type Culture Collection, and they both showed the same property. Furthermore, their growth was strictly dependent on arginine and phenylalanine availability, as determined in yield studies (data not shown).

It is possible that the nonproteolytic type B cultures we examined have undergone mutation, with subsequent loss of proteolytic capacity, but are proteolytic (group I) serotype B in their origin.

In summary, chemically defined minimal media have been developed for group I and II strains of *C. botulinum*. In our laboratory, these media have been useful to accurately study the regulation and function of neurotoxin and proteases in the life cycle of *C. botulinum*. The medium should also be useful in studying the survival of *C. botulinum* and its spores in response to various stresses, including heat, high salt concentration, and extremes of pH and for metabolic and genetic studies in other laboratories.

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